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	For:	**IMPROVED METHOD FOR THE ANALYSIS OF NUCLEIC ACID SAMPLES**

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LETTER RE: PRIORITY

Sir:

Applicant hereby claims priority from Australian Patent Application No. PS 0642 filed on February 20, 2002, through PCT Application No. PCT/AU03/00213, filed on February 20, 2003.

Respectfully submitted,

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I, SMILJA DRAGOSAVLJEVIC, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 0642 for a patent by BIOMOLECULAR RESEARCH SOLUTIONS PTY LTD as filed on 20 February 2002.

WITNESS my hand this
Tenth day of March 2003

S. Dragosavljevic

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ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "Improved method for the analysis of nucleic acid samples"

The invention is described in the following statement:

"Improved method for the analysis of nucleic acid samples"

Field of the Invention

The present invention relates to improved methods for collecting and analysing nucleic acid samples such as nucleic acid samples of forensic value from crime scenes. The present invention also relates to databases containing data obtained using the improved methods.

Background of the Invention

The analysis of DNA and in particular DNA identification is used in many areas of research and commercial activity including agriculture, veterinary science, medicine and forensics. In agriculture and veterinary science, DNA fingerprinting is used to identify plant and animal genotypes for breeding and identification purposes and in medical science DNA fingerprinting is used for various purposes including identification of related individuals.

DNA fingerprinting has also become an important tool in forensic science and law enforcement. In DNA forensics, DNA isolated from crime scenes can be amplified and visualised using techniques such as polymerase chain reaction ("PCR") and gel electrophoresis and the resulting fingerprint can be used to place a suspect at a crime scene.

The methods used to analyse DNA, such as PCR amplification, are simple, quick and highly sensitive so they can be carried out using small samples or samples that have been partially degraded. However, the sensitivity of DNA analysis techniques also has potential disadvantages. For example, strict contamination control is essential when undertaking analysis using PCR as contaminants in the starting sample will also be amplified. This is particularly so when the contaminants are amplicons derived from PCR as these are amplified with high efficiency during PCR. Consequently, forensic and other testing laboratories go to considerable effort to prevent the contamination of samples during both the collection and processing stages.

When samples are contaminated and the source of the contamination can be readily identified (e.g. the person collecting the sample or running the PCR) samples can be taken from those individuals and the PCR bands generated by that individual subtracted from the test results to correct for the contamination. In a crime scene where multiple individuals may be present, most contaminating individuals (samples not belonging to the perpetrator/s) can be identified and the effects of that contamination eliminated from further analysis.

However, to date it has not been recognised that DNA samples could be purposefully contaminated with the intention of confounding future DNA analysis. For example, microsatellite PCR amplicons generated by the use of a commercially available fingerprinting kit on a standard control or random tissue sample (hereafter referred to as "perfect amplicons") could be added to water or another solvent and used to contaminate a sample or the area from which a sample for future DNA analysis is to be taken. In the example of a contaminated crime scene, these perfect amplicons would be collected along with the forensic sample during the collection of samples for forensic analysis from the scene. These perfect amplicons would be efficiently isolated using current DNA extraction methods widely used in DNA forensics and may be present in a vast excess over the DNA of true forensic value from the crime scene. Furthermore, being short perfect amplicons, they would be amplified with greater efficiency than any genomic DNA present. Upon amplification the resulting profile would consist almost entirely of the contaminating perfect amplicon DNA. Depending on the nature of the contaminating amplicons, the resultant profile may be indistinguishable from a real profile, or may render the identification of the genuine profile of the forensic sample difficult or impossible to determine.

Although current forensic testing usually uses PCR amplification of selected microsatellite regions from the forensic sample nucleic acid, other methods in use or development such as mitochondrial DNA sequencing, single nucleotide polymorphism analysis, low copy number PCR and other methods known to those familiar with DNA analysis methods are also susceptible to this form of contamination.

When not accounted for in the testing process, the potential for contamination of this nature compromises the validity of the DNA analysis and substantially limits the strength of any conclusions drawn therefrom. For example, crime scenes could be contaminated with nucleic acid with a view to confounding future forensic analysis and limiting the legal value of the DNA analysis results used in court proceedings.

At present, the techniques used to analyse nucleic acid samples for forensic purposes do not reliably distinguish between nucleic acids added to contaminate the sample and the true target nucleic acids in a sample. Laboratory procedures currently used are designed to minimise contamination of samples in the laboratory and will not be effective in removing contamination when contaminated samples are presented to the laboratory for analysis.

The most common and widely used system to reduce or remove contamination with PCR derived amplicons relies on the incorporation of DNA nucleotide analogues such as deoxy-uracil triphosphate (dUTP) into DNA during PCR amplification. However, this method is also designed to prevent laboratory cross contamination and does not address the problems encountered when a sample has been contaminated with nucleic acid containing deoxy-thymine triphosphate (thymine, dTTP) instead of dUTP. Consequently this method of contamination control is easily avoided and is not effective in removing contamination when dTTP containing samples are presented to the laboratory for analysis.

Thus, the present invention seeks to provide methods that deal with the previously unrecognised problem of reliably detecting the presence of contamination and processing nucleic acid samples that have the potential of being, or have been, purposefully contaminated to remove the contaminant.

Summary of the Invention

The present invention provides a method of analysing a nucleic acid sample obtained from a site comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the site.

The present invention also provides a method of screening a nucleic acid sample for contaminants that have been purposefully introduced into the sample, the method comprising the step of treating the sample to detect the contaminants.

The methods of the present invention may be broadly applied and in particular may be applied to forensics and animal, plant and human nucleic acid testing.

Brief Description of the Drawings

Figure 1 depicts a gel electrophoresis of various PCR amplifications of uncontaminated and contaminated samples; and

Figure 2 depicts a gel electrophoresis of another series of PCR amplifications of treated and untreated contaminated samples.

Detailed description of the Invention

The present invention provides a method of analysing a nucleic acid sample obtained from a site comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the site.

For the purposes of the present invention the phrase "contaminating nucleic acid/s" is defined as nucleic acid that has been introduced to a site or a sample to confound future analysis of target nucleic acids present at the site or in the sample. The contaminating nucleic acid may be cell bound, free or substantially free from other cell components and may be deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein nucleic acid (PNA) or any other nucleic acid containing composition that is capable of detection during testing procedures.

When the contaminating nucleic acid is free or substantially free from other cell components it may be in a form that is particularly well adapted for amplification via PCR or some other amplification process that is used in forensic analysis. One particular example of this type of contaminating nucleic acid is an amplicon derived from a PCR or another DNA amplification process and in particular a

degradation resistant amplicon that has been specifically designed to persist at a site or in a sample. Synthetic DNA, RNA or PNA may also be used.

The contamination addressed by the present invention may confound any nucleic acid analysis protocol where samples may be contaminated. Thus, while specific mention is made herein of PCR, it will be appreciated that the same contamination could be used to alter the results of other analysis methods such as, but not limited to, mitochondrial DNA sequencing, single nucleotide polymorphism (SNP) analysis and low copy number PCR.

When the contaminating nucleic acid is cell bound it may also be in a form that is particularly well adapted for amplification via PCR or some other amplification process that is used for analysing nucleic acids. One particular example of this type of contaminating nucleic acid is a bacterial preparation where the bacteria have been engineered to contain one or more multicopy plasmids each comprising one or more amplicons able to be amplified during standard forensic PCR processes.

The pre-treatment may be varied depending on the nature of the contaminating nucleic acids that require removal or inactivation. Thus, when the contaminating nucleic acids are free or substantially free from other cell components, the pre-treatment may comprise treating the sample to preferentially remove or inactivate nucleic acids that are free or substantially free from other cell components. Such treatments may be one or more treatments selected from the group comprising: (i) enzymic treatments such as contacting the sample with enzymes that preferentially breakdown free nucleic acids e.g. DNAses, RNAses, exonucleases and endonucleases; (ii) physical treatments that remove free contaminating nucleic acid from the sample based on differences between physical characteristics of the contaminating nucleic acid and the target nucleic acid such as charge, density, weight and size and the actual techniques used may be selected from the group comprising centrifugation, washing, filtration and chromatography such a gel filtration chromatography; or (iii) chemical treatments such as the use of sodium hydroxide, sodium hypochlorite, detergents (e.g. Tween 20, Alcanox or SDS) as well as proprietary products designed to remove

nucleic acids form surfaces such as DNA Zap, RNA Zap, DNA Free or RNA Free (Ambion Inc., Austin, Texas, USA).

When the contaminating nucleic acids are free from other cell components then the pre-treatment may comprise contacting the sample with nucleic acid probes that preferentially bind to the contaminating nucleic acids and render them removable from the sample. This is particularly appropriate for the removal of contaminating nucleic acids in the form of PCR derived amplicons.

Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site comprising the step of contacting the sample with a nucleic acid probe that preferentially binds to the contaminating nucleic acids and renders them removable from the sample.

The choice of probe is entirely dependent on the nature of the contaminant. However, it is envisaged that the most common contaminants will be derived from the commercially available forensic DNA test kits and in particular the positive controls that can be readily amplified via PCR. Thus, the probes may be designed to specifically hybridise to the amplification product of the positive control from a proprietary kit. In the event that a new contaminant is produced then it would be necessary to first characterise the contaminant to enable appropriate probes to be designed for use in the method.

The nucleic acid probe may be labelled to aid in its removal from the sample. Suitable labels include biotin/streptavidin.

In one particular form of the invention the contaminating nucleic acid bound to the labelled probe is removed through the use of a chromatography column adapted to specifically bind the label.

When the contaminating nucleic acids are cell bound or otherwise cell associated in a way that prevents or hampers their removal or inactivation, such as if contained in bacterial cells, then additional pre-treatments may be required. For example, when the contaminating nucleic acids are contained within bacterial

cells, an additional step to selectively lyse the bacterial cells may be employed. Once the bacterial cells have been lysed the techniques discussed above could be used to complete the pre-treatment. Contaminants in the form of bacterial cells may also be removed by using a filter that selectively removes the bacterial cells from the sample.

Once the sample has been pre-treated it can be treated according to standard techniques for nucleic acid analysis. Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site comprising the steps of:

(i) pre-treating the sample to remove or inactivate contaminating nucleic acids originating from the site; and

(ii) characterising the target nucleic acids in the sample.

The nucleic acids in the sample can be characterised by any one of a range of techniques that are presently in use in the field. These techniques generally involve isolating the target nucleic acid and then treating it such that it can be conveniently characterised. These techniques and procedures are well known by those skilled in the art.

The target nucleic acid may be isolated using standard extraction protocols that involve lysing the cells to free the nucleic acid and then separating the nucleic acid from other cellular material. Once isolated, to increase the amount of target nucleic acid, the target nucleic acid may be selectively amplified using PCR or some other technique that is able to replicate the target DNA to increase the amount available for further analysis. Once amplified the target nucleic acid can be visualised using gel electrophoresis. Proprietary DNA fingerprinting kits can also be used to perform this part of the method.

Screening Methods

Rather than applying the method of the present invention to all samples taken for nucleic acid analysis, it may be preferred to screen samples for contamination prior to nucleic acid analysis. By applying this method, samples that have been contaminated can be identified and handled accordingly.

Thus, the present invention also provides a method of screening a nucleic acid sample for contaminants that have been purposefully introduced into the sample, the method comprising the step of treating the sample to locate the contaminants.

Various treatments may be applied to a sample to screen for contaminants including the use of a detectable probe designed to selectively hybridise to the contaminant. As indicated above, it is expected the most common contaminants will be sourced from commercial DNA analysis kits so the design of probes for this purpose will be routine to those skilled in the art. Alternatively, the wash solutions, filtrate, chromatography column eluate or other products resulting from the procedures used to remove potential contaminants could be tested for the presence of the contaminants.

Databases

The method of the present invention allows for the accurate identification of nucleic acids and counters the effects of contaminants that may have been introduced into a sample with a view to confounding their analysis. DNA fingerprint databases currently in existence include fingerprints that have been determined using methods that do not account for the potential problems of contamination. Given the possibility of contamination, the conclusions drawn from fingerprints in the current databases may be queried. This could be a particular problem in court proceedings where DNA fingerprint evidence has been used to identify a perpetrator. It is possible that DNA analysis performed with protocols that do not account for purposeful contamination may be held inadmissible.

Thus, the present invention also provides a database comprising the results of at least one analysis generated from a method according to the present invention, such as DNA fingerprint.

Preferably, the database is computerised for ease of use and comprises fingerprints of known perpetrators. However, the database can contain any data obtained through the use of the method of the present invention.

Kits

The method of the present invention may be conveniently performed using a kit comprising a series of reagents necessary to carry out the method. Thus, the present invention also provides a nucleic acid analysis kit comprising a means to remove a nucleic acid contaminant from a sample to be subjected to analysis.

The means may be varied and includes those discussed herein such as labelled probe adapted to bind to the contaminant and thus aid in its removal. Alternatively, the means may comprise an enzyme or chemical that can be added to the sample and inactivate of remove the contaminant preferentially or selectively relative to the target nucleic acid.

The method of the present invention is generally applicable to methods for identifying or analysing nucleic acid samples. Described hereunder, are particular applications that demonstrate the broad application of the present invention.

Forensics

The method of the present invention may be of particular use in the analysis of target nucleic acid obtained from crime scenes. Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site in the form of a crime scene comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the crime scene.

For the purposes of the present invention the phrase "crime scene" is defined to include sites where a crime has been committed or other sites away from the crime scene, where nucleic acid of forensic value relevant to the crime may be found.

In addition to carrying out the method of analysis described above in relation to samples taken from a crime scene it may also be useful when assessing crime scenes to screen the crime scene for contaminating nucleic acid. To date, as this potential problem has not been recognised no specific screening takes place.

Thus, the present invention also provides a method of analysing a crime scene comprising the step of screening the crime scene for contaminating nucleic acids.

The screening step may be carried out by any means apparent to those skilled in the art for detecting contaminating nucleic acids at a crime scene. One such technique involves taking a sample from a point in the crime scene that would not normally contain nucleic acids. This form of screen is particularly appropriate for locating contaminating nucleic acids, such as amplicons, that have been sprayed in liquid form at a crime scene.

In such situations contamination of crime scenes by perfect amplicon material can be detected by testing samples collected from areas within the crime scene surrounding areas in which target nucleic acids are located e.g. sections of walls or floors adjacent a blood stain. The generation of significant DNA profiles from such areas that would not be expected to contain high levels of DNA may indicate a contaminated crime scene.

In situations where individual tissue samples (eg blood, hair etc) have been contaminated by perfect amplicons, contamination may be detected by hybridisation with microsatellite probes or amplification with primers that bind to the primer sequences of known fingerprinting kits, under conditions such that the cells in the tissue sample are not disrupted sufficiently to release significant amounts of genomic DNA (e.g. reduced temperatures).

Veterinary

The method of the present invention may also be applied to the analysis of target nucleic acid obtained from animals. Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site in the form of an animal comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the crime scene.

Nucleic acid samples from animals are analysed for a wide range of purposes. Animals of a particular species or breed may be assessed by a potential buyer or breeder to confirm their genotype. Furthermore, commercial herds or products therefrom such as meat may require analysis to assess if they qualify for a government sponsored subsidy or that they meet certain regulatory requirements, such as GMO's or quarantine standards. In all of these situations there is a motive for a person to tamper with the samples for monetary or some other gain.

Agriculture

The method of the present invention may also be applied to the analysis of target nucleic acid obtained from plants. Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site in the form of a plant such as a seed comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the crime scene.

Nucleic acid samples from plants are analysed for a wide range of purposes. Plants of a particular species or variety may be assessed by a potential buyer or breeder to confirm their genotype. Furthermore, commercial crops or products therefrom may require analysis to assess if they qualify for a government sponsored subsidy or that they meet certain regulatory requirements, such as GMO's or quarantine standards. In all of these situations there is a motive for a person to tamper with the samples for monetary or some other gain.

Parentage Testing

The method of the present invention may also be applied to the analysis of target nucleic acid obtained from humans for assessing parentage. Thus, the present invention also provides a method of analysing a nucleic acid sample obtained from a site in the form of a human comprising the step of pretreating the sample to remove or inactivate contaminating nucleic acids originating from the crime scene.

There are obvious motives for a person to tamper with a sample taken for assessment of parentage. Whilst the mechanics of tampering with samples for this purpose may be slightly for complicated, the method of the present invention also solves the problems that could lead to the abuse of this form of nucleic acid testing.

The invention will now be described with reference to two examples. The description of the examples is in no way limiting on the more general description of the invention in the preceding paragraphs.

Examples

Example 1 – Demonstration of PCR amplification of contaminating microsatellite PCR products during amplification of genomic DNA microsatellite loci.

Materials and Methods:

Template genomic DNA was isolated from muscle tissue of feral cat GD450. This cat carries alleles 25/21 at the locus FCA 69H (GenBank AF130500) carried by the cat chromosome B4. Genomic DNA was extracted from 4.5 mg of muscle tissue using the MasterPure™ DNA Purification Kit (Epicentre Technologies, Madison, WI, US). The manufacturers recommended protocol for tissue extraction was followed with the exceptions that digestion of the tissue sample with Proteinase K was carried out overnight at 65°C and protein was pelleted by centrifugation at 4°C.

Three different contaminants were produced by PCR amplification of selected microsatellite loci from genomic DNA isolated from tissue of 3 feral cats:

Cat MV1 (alleles 25/11): allele 25 in common with GD450

Cat MV4 (alleles 31/21): allele 21 in common with GD450

Cat MV5 (alleles 27/11): no allele in common with GD450

The contaminant microsatellite PCR products were approximated to have a final concentration of 50 ng/µl.

PCR conditions and primer sequences used in this study are given in Menotti-Raymond, M.; David, V.A.; Lyons, L.A.; Schäffer, A.A.; Tomlin, J.F.; Hutton, M.K.; O'Brien, S.J. (1999). A Genetic Linkage Map of Microsatellites in the Domestic Cat (*Felis catus*). Genomics **57** (1), 9-23. Medline 99208656.

Reactions containing 1µl of GD450 genomic DNA and various dilutions of single or mixed contaminant microsatellite PCR products were prepared as described in Table 1.

Table 1

Gel lane	Genomic DNA Vol.	Contaminant	Contaminant dilution (template Vol.)	Comments
1	1 ul	None	n/a	GD450 Positive control
2	1 ul	MV1	10^{-15} (1 ul)	GD450 only
3	1 ul	MV1	10^{-12} (1 ul)	GD450 only
4	1 ul	MV1	10^{-9} (1 ul)	GD450 only
5	1 ul	MV1	10^{-6} (1 ul)	GD450/MV1 mixed profile
6	1 ul	MV1	10^{-3} (1 ul)	GD450/MV1 mixed profile
7	1 ul	MV1	1 (1 ul)	GD450/MV1 mixed profile
8	1 ul	MV4	10^{-15} (1 ul)	GD 450 only
9	1 ul	MV4	10^{-12} (1 ul)	GD450 only
10	1 ul	MV4	10^{-9} (1 ul)	GD 450 only
11	1 ul	MV4	10^{-6} (1 ul)	GD450 only
12	1 ul	MV4	10^{-3} (1 ul)	GD450/MV4 mixed profile
13	1 ul	MV4	1 (1 ul)	GD450/MV4 mixed profile
14	1 ul	MV5	10^{-15} (1 ul)	GD450 only

15	1 ul	MV5	10^{-12} (1 ul)	GD450 only
16	1 ul	MV5	10^{-9} (1 ul)	GD450 only
17	1 ul	MV5	10^{-6} (1 ul)	GD450/MV5 mixed profile
18	1 ul	MV5	10^{-3} (1 ul)	GD450/MV5 mixed profile
19	1 ul	MV5	1 (1 ul)	GD450/MV5 mixed profile
20	1 ul	Lanes 20-25 Mix of: MV1 + MV4 + MV5	10^{-15} (1 ul each)	Amplification failure
21	1 ul		10^{-12} (1 ul each)	Amplification failure
22	1 ul		10^{-9} (1 ul each)	Amplification failure
23	1 ul		10^{-6} (1 ul each)	Amplification failure
24	1 ul		10^{-3} (1 ul each)	GD450/MV1/MV4/MV5 profile
25	1 ul		1 (1 ul each)	Too much template
26	none	none	n/a	No DNA Negative control

Results

The results of PCR amplification of various mixtures of cat GD450 genomic DNA and microsatellite PCR product contaminants (see Table 1) are shown in Figure 1. Short alleles (few repeats) give a stronger fluorescent signal if the template is a PCR product possibly due to increased efficiency of amplification.

All samples tested contained equivalent amounts of genomic DNA from cat GD450 and various amounts of contaminant PCR product generated from one or more cats MV1, MV4, MV5 (Table 1). PCR amplification of genomic cat GD450 DNA alone generated the expected bands for alleles 25 and 21 (Figure 1, lane 1). In the presence of low concentrations of the contaminant (10^{-15} – 10^{-9}) the GD450 profile was the only profile present. At higher concentrations (10^{-6}, 10^{-3}, undiluted) both the GD450 and the contaminant profile were present, making it difficult, or impossible, to determine the correct GD450 profile.

In mixtures containing MV1 or MV5 microsatellite PCR products (Lanes 2-7, and 14-19, respectively) the contaminant profile was evident when undiluted PCR (Lanes 7 and 19, respectively) product was added as well as dilutions of 10^{-3} and 10^{-6} (Lanes 5, 6, and 17, 18, respectively). When MV4 was the source of the contaminant it was detected in only undiluted and 10^{-3} samples (Lanes 12 and 13). When all three contaminating PCR products were mixed (total of 3µl of PCR

product added) the amount of contaminant appeared to inhibit the PCR amplification (Lane 25) but at reduced concentrations (Lane 24) the profile consisted of a combination of the three individual profiles. In lane 24, the alleles (11 and 25) present in more than one template (MV1+MV5 and MV1+GD450, respectively) give stronger signals than alleles 31, 27, 21 present in only one template due to the presence of multiple products.

These results clearly demonstrate that contaminating microsatellite PCR products are efficiently amplified during subsequent amplification for microsatellites from genomic DNA of cat GD450. In some of these mixtures subsequent PCR amplification resulted in a combination of the individual profiles such that the correct profile of test genomic DNA from cat GD450 is effectively masked. There is a clear relationship between the amount of contaminating microsatellite PCR product added to the genomic DNA and the amplification of the contaminant, with less contaminant resulting in reduced amplification of the contaminant in subsequent PCR amplifications. The amount of contaminant required to achieve this masking effect is extremely small. The undiluted contaminant had a concentration of approximately 50ng/µl. At a dilution of 10^{-6} only 50 femtograms of PCR product was present. Since a typical PCR reaction would contain approximately 1ng –100ng of genomic DNA it is clear that only trace amounts of contaminating PCR product are required to mask the genuine GD450 profile.

Example 2 – Demonstration that contaminating microsatellite PCR products are extracted with genomic DNA and efficiently amplified during amplification of genomic DNA microsatellite loci.

Materials and Methods

Template genomic DNA was isolated from muscle tissue of feral cat. Genomic DNA was isolated from cat GD450 that scores 25/21 at the locus FCA 69H (GenBank AF130500) carried by the cat chromosome B4 or from feral cat MV5 which gives 27/11 at the same locus. The contaminant for use in these experiments was produced by PCR amplification of selected microsatellite loci

from DNA of cat MV5 and diluted with water to give a final concentration of 20 ng/µl.

PCR conditions and primer sequences used in this study are given in Menotti-Raymond, M.; David, V.A.; Lyons, L.A.; Schäffer, A.A.; Tomlin, J.F.; Hutton, M.K.; O'Brien, S.J. (1999). A Genetic Linkage Map of Microsatellites in the Domestic Cat (*Felis catus*). Genomics **57** (1), 9-23. Medline 99208656.

Extraction of genomic DNA and contaminant removal

The genomic DNA of the cat GD450 was extracted from 4.5 mg of muscle tissue with the MasterPure™ DNA Purification Kit (Epicentre Technologies, Madison, WI, US) using the modified protocol described in Experiment One. The Masterpure kit provided a more stringent DNA extraction method than the phenol or Chelex™ extraction methods recommended in forensic kits such as the AmpFISTR Profiler Plus™ Kit from Applied Biosystems and should result in less carry through of any contamination during the genomic DNA extraction procedure.

Seven different reaction tubes were setup:

1. Tissue (cat GD450) alone to obtain non contaminated genomic DNA profile
2. 5 ul (100ng) of cat MV5 contaminant DNA alone
3. Tissue (cat GD450) + 5 ul (100ng) of MV5 contaminant
4. Tissue (cat GD450) + 5 ul (100ng) of MV5 contaminant digested with *Hae* III (Promega)
5. Tissue (cat GD450) + 5 ul (100ng) of MV5 contaminant digested with DNAse I (Sigma)
6. Tissue (cat GD450) + 5 ul (100ng) of MV5 contaminant treated with DNAZap (Ambion)

7. Tissue (cat GD450) + 5 ul (100ng) of MV5 contaminant washed with water.

In seven 1.5 ml microfuge tubes, cat GD450 tissue and/or cat MV5 microsatellite PCR product contaminant were mixed and left in contact for 30 min at room temperature. In tubes 1, 2, and 3 no further treatment was performed. In tube 4, 10X Promega Buffer B (3µl), 0.5µl Promega *Hae* III restriction enzyme (9 units) and 21.5µl of water were added and incubated at 37°C for 1 hour. In tube 5, 10X Promega Buffer C (3 µl), 0.5µl DNAse I (~50 units) and 21.5µl water were added and incubated at 37°C for 1 hour. In tube 6, DNAZap Solution 1 (10µl) was added to the tube immediately followed by 10µl of DNAZap Solution 2 (Ambion Pty Ltd). After approximately 10 seconds the tissue sample was thoroughly rinsed with deionised water prior to further use. In tube 7, the tissue sample was washed twice 1 ml of water, dried with tissue paper and transferred into a new tube. The tissue was then washed again with 2 x 1 ml of water.

In every tube, the integrity of the tissue samples was preserved following the treatments.

Following the above treatments 300µl of Masterpure Tissue and Cell Lysis Buffer + 50µg Proteinase K were added (Epicentre Technologies). The samples were incubated overnight at 65°C in a hybridization oven with rotation. After this treatment, no tissue was left in the tubes. RNA was removed by addition of 5µg of RNAse A and incubation at 37°C for 30 min. Tubes were cooled on ice and 150µl of MPC Protein Precipitation Reagent were added (Epicentre Technologies). The precipitate was pelleted by centrifugation at 10,000g for 10 minutes at 4°C. Supernatants were transferred into new tubes.

After addition of 500µl of isopropanol and centrifugation for 10 minutes, the DNA pellet was washed with 70% EtOH and resuspended in 40µl of water.

<u>PCR reactions</u>

Samples from tubes 1 –7 were used in individual PCR amplifications to detect microsatellite loci from cat GD450. Amplifications of individual microsatellite loci were performed in either 10µl or 20µl reactions containing 1µl of template nucleic acid solution from each of tubes 1-7 according to the procedure of Menotti-Raymond *et al* (1999). The 10µl reactions were approximated to contain 2.5ng of contaminant (1/40 x 100ng) whilst the 20µl reactions contained approximately 10ng of contaminant (4 x 2.5ng).

A PCR amplification control was performed where 1ul of contaminant (20ng) was reamplified as above.

Results

The results (Figure 2) demonstrate the presence of the substantially correct profiles for MV5 contaminant and GD450 controls (Contaminant and Sample 1 lanes, respectively) as well as animal positive controls (animals A, B and C). Significant stutter peaks were present in the MV5 contaminant controls equivalent to alleles 25, 23 and 9. Additionally the MV5 contaminant control contained a band at a size equivalent to an allele at 22. The exact identity of this band is unknown but it is possibly the result of heteroduplex formation during PCR. In the GD450 positive control stutter peaks at allele equivalent 23, 19 and 17 are present. Smaller are bands in GD450 at allele equivalents 11 and 9 are the result of spillage from adjacent lanes.

In samples containing mixtures of cat GD450 genomic DNA and MV5 microsatellite PCR product contaminant (mixture samples) only bands representative of the MV5 contaminant were present. Since the contaminant amplified in these samples was added to cat GD450 tissue prior to genomic DNA extraction, the results show that contaminating microsatellite PCR products were efficiently extracted during the isolation of DNA from tissue of cat GD450. These PCR product contaminants could be amplified during subsequent PCR implication for cat GD450 microsatellite loci. The results further show that when 100ng of contaminating microsatellite was added to 4.5mg of tissue the contaminating

microsatellite PCR products were able to entirely mask the genuine cat GD450 profile (eg: mixture samples).

With the exception of DNAse (mixture + DNAse) the treatments trialled to remove the contaminating microsatellite PCR products failed to have any significant effect on the level of contaminant amplified during subsequent amplification for cat GD450 microsatellite loci. In the sample treated with DNAse I, however, the microsatellite contaminant was efficiently removed since no detectable cat MV5 bands were present following PCR amplification for cat GD450 microsatellite loci (mixture + DNAse I, 1μL and 5μL samples). However, there was also an absence of GD450 bands suggesting that the treatment used also completely removed genomic DNA from the sample.

This result demonstrated that the removal of contaminating microsatellite PCR products from tissue is possible. However, it is not easily achieved by either physical or chemical methods that are frequently used to remove contaminating DNA. It also showed that the complete removal of contaminating microsatellite PCR products requires additions/modifications to both reagents and protocols in DNA extraction methods often used for DNA fingerprinting studies. DNAse I was effective at removing contaminating microsatellite PCR products but the method used in this study is not suitable for inclusion in a DNA extraction kit.

Throughout the specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Figure 1

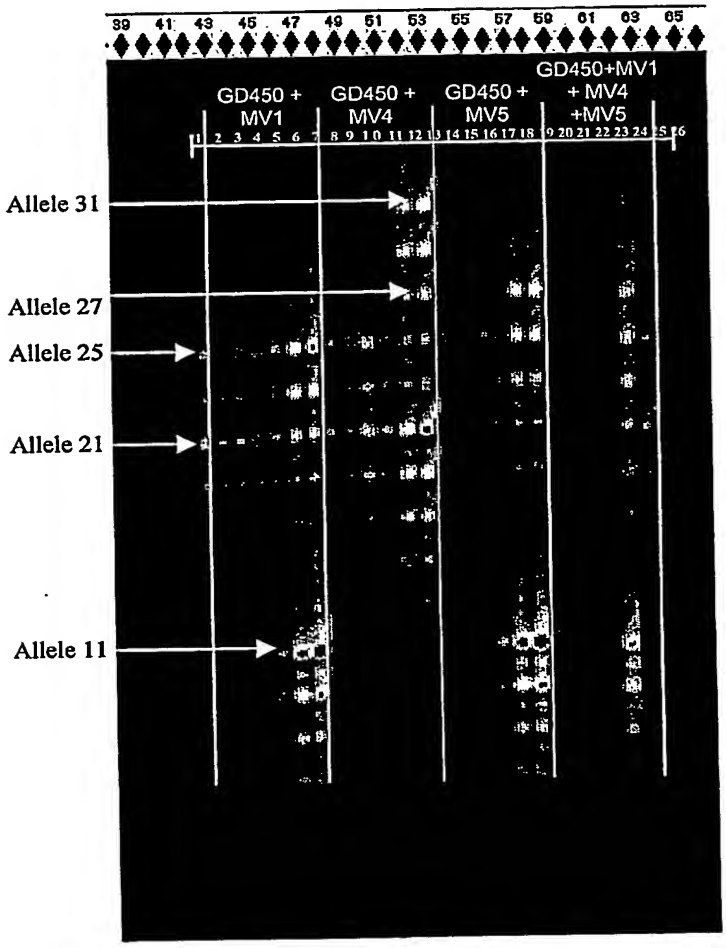
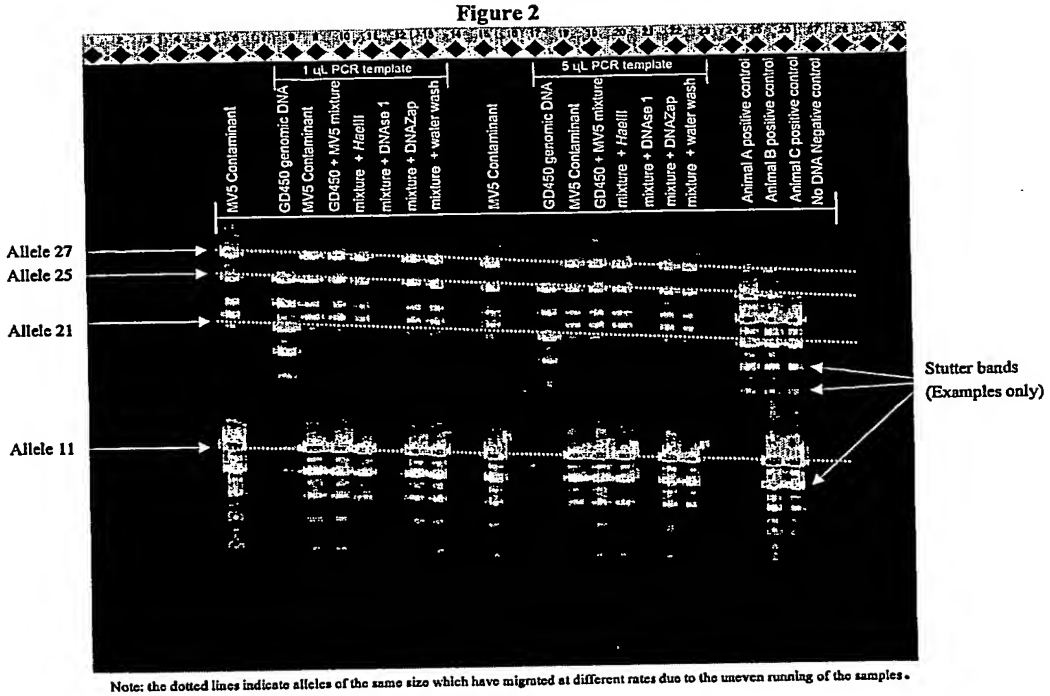


Figure 2



Note: the dotted lines indicate alleles of the same size which have migrated at different rates due to the uneven running of the samples.